Further Evidence of *Mycobacterium tuberculosis* in the Sputum of Culture-Negative Pulmonary Tuberculosis Suspects using an Ultrasensitive Molecular Assay.

Guillermo Madico, MD, PhD [a]; Solange Vinhas, PhD [b]; Beverley Orr, MT (ASCP) [c]; Laura White, PhD [d]; Mary Gaeddert, MPH [a]; Nancy S. Miller, MD [c,e]; Moses Mpeirwe MSc [f]; Patrick Orikiriza, MSc [f,g]; Juliet Mwanga-Amumpaire, MBChB [f]; Yap Boum II, PhD [f,g]; Moises Palaci, PhD [b]; Reynaldo Dietze, MD, PhD [b]; Edward C. Jones-López, MD, MSc [a,h].

[a] Section of Infectious Diseases, Department of Medicine, Boston Medical Center and Boston University School of Medicine, Boston, Massachusetts, U.S.A.
[b] Núcleo de Doenças Infecciosas, Universidade Federal do Espírito Santo, Vitória, Brazil.
[c] Clinical Microbiology Laboratory, Boston Medical Center, Boston, Massachusetts, U.S.A.
[d] Department of Biostatistics, Boston University School of Public Health, Boston, Massachusetts, U.S.A.
[e] Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, Massachusetts, U.S.A.
[f] Mbarara University of Science and Technology, Mbarara, Uganda.
[g] Epicentre, Médecins sans Frontières, Mbarara, Uganda.
[h] Quest Diagnostics Infectious Diseases, San Juan Capistrano, California, U.S.A (current affiliation).

Author emails (in authorship order):
gmadico@bu.edu
svinhas@ndi.ufes.br
Beverly.Orr@bmc.org
lfwhite@bu.edu
gaeddert@bu.edu
Nancy.Miller@bmc.org
mpeirwemoses@yahoo.com
Patrick.ORIKIRIZA@epicentre.msf.org
Juliet.MWANGA@epicentre.msf.org
Yap.BOUM@epicentre.msf.org

mpalaci@ndi.ufes.br
rdietze@ndi.ufes.br
eddie.mex@gmail.com

Corresponding author:
Edward C. Jones-López, MD, MSc
Affiliation address:
Section of Infectious Diseases
Boston University Medical School and
Boston Medical Center
801 Massachusetts Ave, Crosstown 2009
Boston, MA 02118

Present address:
Medical Director, Infectious Diseases, R&D
Quest Diagnostics Infectious Diseases
33608 Ortega Highway
San Juan Capistrano, CA 92675
Phone: (949) 728 4040
Fax: (949) 728 7180
Email: eddie.mex@gmail.com

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Running Title: Diagnosis of paucibacillary TB disease

Declarations of interest: Drs. Madico and Jones-López are co-inventors of TOP TB assay's primers and probes. This invention is owned by Boston Medical Center, which has submitted an international patent application. Dr. Madico, Dr. Jones-López and Mr. Joe Straight were co-founders and shareholders of Thisis Diagnostics Inc., a start-up company created in 2013 to develop and commercialize the TOP TB assay. Thisis Diagnostics Inc. was dissolved in late 2018.

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Abstract:

Background: Rapid diagnosis of pulmonary tuberculosis (TB) is critical to TB control. However, many patients with paucibacillary TB disease remain undiagnosed. Current TB elimination goals require new tools to diagnose early disease. We evaluated performance of the Totally Optimized PCR (TOP) TB assay, a novel ultrasensitive molecular test.

Methods: We assessed analytical specificity against nontuberculous mycobacteria (NTM), and estimated the diagnostic accuracy of TOP in a pilot study in Brazil (n=46) and a cross-sectional study in Boston (n=60). We compared TOP results to culture and a composite reference standard (CRS).

Results: TOP exhibited no cross-reactivity against NTM. We tested 132 respiratory specimens from 106 patients with suspected pulmonary TB. The pilot demonstrated feasibility and 100% (95% CI 85-100) sensitivity in predominantly smear-positive specimens; TOP's specificity against solid media culture was low (58%, 37-77) but improved against a CRS (93%, 68-100). Similarly, when using the CRS in the Boston study, TOP (88%, 1-99) had greater sensitivity than solid or liquid media culture (25%, 3-65) and similar specificity (both 100%, 93-100).

Conclusions: The TOP assay enables detection of *M. tuberculosis* in culture-negative paucibacillary disease. While the use of TOP for the diagnosis of paucibacillary disease will require further clinical validation, its high sensitivity indicate a more immediate utility to rule out TB.

Keywords: *Mycobacterium tuberculosis*; paucibacillary disease; diagnosis; culture-negative disease; genotyping

Introduction

Tuberculosis (TB) has surpassed HIV/AIDS as the leading cause of death by an infectious disease worldwide and remains a major threat to global health, productivity, and socioeconomic development [1]. Despite recent advances in diagnostic test development [2], it is estimated that at least one-third of individuals with TB disease are never diagnosed. This leads to poor outcomes and uninterrupted *Mycobacterium tuberculosis* transmission [3]. A 20% annual decline in the global TB incidence will be required to achieve the World Health Organization (WHO) stated goal of eliminating TB by 2050 [4, 5], but the incidence is now decreasing by less than 2% per year. Improved diagnostics are needed to bridge this gap.

As characteristically seen in HIV-infected patients and children, the burden of undiagnosed disease is unevenly concentrated in so-called "special populations" [6]. This term is reserved for patients with a low bacterial count in sputum (e.g. paucibacillary TB disease). A key obstacle to augmenting case detection is that current sputum-based technologies establish a diagnosis at a relatively late stage (i.e. when disease severity is sufficiently advanced for mycobacterial growth in culture media). Despite their elevated cost, unacceptably long turn-around time, and need for laboratory biosafety containment, mycobacterial cultures remain the reference method for TB diagnosis because they: 1) remain the most sensitive diagnostic test available, 2) can demonstrate bacterial viability, and 3) allow for phenotypic drug susceptibility testing. However, culture yields suboptimal detection, particularly for patients with paucibacillary disease [7]. Historically, evidence of widespread culture-negative TB disease was reported in autopsy studies of HIV-infected persons in sub-Saharan Africa, pediatric TB investigations, and household contact studies [2, 6]. More recently, evidence of active TB disease caused by non-culturable organisms has been significantly expanded using advanced imaging techniques [8] and enriched mycobacterial culture media [9, 10], considerably complicating the diagnostic landscape [11].

Newly developed molecular assays significantly shorten the "sample-to-treatment" cascade of care and approach the sensitivity of culture. However, these tests are still best suited for detection of TB at later stages and have had limited impact in improving treatment outcomes [12-14]. These data suggest that a marked improvement in patient outcomes will likely require shifting diagnostic emphasis to earlier detection –i.e. before culture positivity [15]. However, efforts towards this goal are stymied by the continued use of cultures as the gold standard for

TB diagnosis [6, 16, 17] and the associated methodological challenges that are required to overcome this limitation [18-21].

We recently reported the diagnostic performance of a new nucleic acid amplification test the "Totally Optimized PCR (TOP) TB assay" in a cohort of HIV-infected patients with suspected pulmonary TB in Mbarara, Uganda [22]. Our results demonstrated that TOP enabled detection of *M. tuberculosis* DNA in the sputum with an estimated 2-3 fold heightened sensitivity relative to liquid mycobacterial cultures and Xpert MTB/RIF (Cepheid, Sunnyvale, CA), when compared to a composite reference standard that included *M. tuberculosis* sequencing. However, the generalizability of our initial study was limited because it included a single site, and we tested a convenience sample of sputum specimens processed for Xpert MTB/RIF. Furthermore, we did not provide sufficient data regarding the assay's analytical specificity against nontuberculous mycobacteria (NTM). In the present report, we provide additional evidence for the latter and show the diagnostic performance of TOP in two additional clinical studies.

Methods.

Assessment of analytical specificity. To determine the analytical specificity of TOP against closely related mycobacteria, we sourced 20 species of NTM (provided by Richard Wallace, MD, University of Texas at Tyler, TX) that were selected because of their genetic proximity to TOP TB target primers.

Clinical studies. We evaluated the diagnostic performance of the TOP TB assay in discarded respiratory samples sourced from TB suspects undergoing a routine clinical evaluation at two clinical sites. We first performed a pilot study in Vitória, Brazil, to demonstrate feasibility and to evaluate assay performance in mostly sputum acid-fast bacilli (AFB) smear-positive specimens. We also conducted a study in hospitalized and ambulatory patients attending a large tertiary care hospital in Boston, Massachusetts. Except for Brazilian specimens where the AFB smear result was used to determine study eligibility, study personnel were blind to routine TB results; coded results were later linked via a study identification number. When available, we used clinical and microbiological information collected as part of routine care (e.g. out of study) to resolve discrepancies between culture and TOP results, and to prepare clinical vignettes (Supplementary Table 4).

Vitória, Brazil. The pilot study was conducted at the Núcleo de Doenças Infecciosas (NDI) in Vitória, the capital city of the State of Espírito Santo. The NDI has organized a network of five laboratories in the metropolitan region of Vitória that serves 16 TB clinics. With approximately 1,400 cases/year, the TB incidence in Espírito Santo is 38/100,000 inhabitants. The prevalence of HIV infection in Espírito Santo is <1% in the general population, and 7% in TB cases [23]. The NDI network performs universal solid culture (Ogawa-Kudoh media) testing on an average of 12,000 samples per year, of which 5-8% are culture-positive (700 to 850 new TB cases per year). Of these, approximately 80% are sputum smear AFB-positive and 20% are AFB-negative.

Boston, Massachusetts. This study was conducted at the Boston Medical Center (BMC) Clinical Microbiology Laboratory. BMC is a private, not-for-profit, 496-bed, academic medical center (www.bmc.org) that serves as the principal safety net hospital for the greater Boston area (57% of patients are from under-served populations and 32% do not speak English as their primary language). Each month, approximately 150-200 (90-150 respiratory) samples are tested for *M. tuberculosis* at the BMC laboratory, of which ~2% are culture-positive and reported to the Boston Public Health Commission (BPHC). BMC identifies an average of 2.6 new cases of TB per month (range 0-5), corresponding to 50-65% of notified TB cases in Boston. Over the three-year period preceding the study (2011-2013) [24], BPHC reported 40-45 new cases per year (incidence rate of 6.5 to 7.1 per 100,000 population) of which 60-70% were sputum smear AFB-negative/culture positive or AFB-negative/culture-negative; 65% had pulmonary TB, 80% were non-US born, 8-9% were HIV infected, and the majority (65-77%) were diagnosed while hospitalized [24].

Laboratory assessments.

Both participating laboratories are state-of-the-art facilities with permanent trained personnel, established quality assurance and quality control protocols, and extensive experience in both TB clinical care and research. There were minor differences between sites in specimen handling prior to TOP testing and culture methods (Supplementary Table 1). Both participating laboratories followed standard mycobacterial laboratory methods as part of routine testing (see Supplementary Data: *Standard Laboratory Methods*).

Sample handling prior to TOP testing. In both laboratories, once the routine work was completed, if the remaining volume of the sample was ≥1 mL, it was coded and stored unprocessed in a refrigerated cabinet until for up to 1 week (as per routine clinical care). In Brazil, extracted DNA after TOP processing was frozen at -80^{o}C for two months until TOP testing in a single batch at the NDI without knowledge of culture results. In Boston, eligible samples were picked up weekly and subjected to the TOP sample processing method within 24-48 hours after receipt in the research laboratory. TOP testing was done in weekly batches without knowledge of smear and cultures results.

TOP TB assay. A detailed description of TOP TB assay methods, including primer and probe design, sample processing and DNA extraction, PCR amplification and amplicon detection has been published [22]. Briefly, the assay targets a gene (*ponA1*) involved in the assembly of peptidoglycans in the *M. tuberculosis* bacterial wall [25]. The assay's diagnostic primer set (3-ponA-F/R) targets sequences unique to all species in the *M. tuberculosis* complex. Amplicons generated by 3-ponA are detected using a capture-probe colorimetric assay, and the resultant Optical Densities (OD) provide a semi-quantitative measurement of bacillary load [22, 26]. To establish the presence of *M. tuberculosis* DNA, specimens that were detected positive using the diagnostic primer set (3-ponA) were confirmed with a second primer set (2-ponA-F/R) used for genotyping. 2-ponA PCR products were sequenced (Genewiz, NJ, USA) to distinguish among five possible genetic variants of *M. tuberculosis* (0^{T}, 1^{T}/1, 2, 3 and 4) that we have correlated to other widely accepted molecular genotyping methods (RFLP, Spoligotyping, SNiPs), as previously described [22].

Analytical strategy. We report the results according to the Standards for Reporting of Diagnostic Accuracy (STARD) guidelines [27, 28]. We calculated the diagnostic TOP OD cut-off for each study separately to account for the inherent variability of the ELISA-type colorimetric detection method used. We calculated the diagnostic cut-off for TOP OD using a cut-off value of three standard deviations above the mean of the OD values of negative controls (e.g., laboratory cut-off) [29], after validating the latter method using receiver operating curve (ROC) tools in a prior study [22]. For the Brazilian pilot study that was biased toward mostly smear AFB+ samples, we first performed a per-specimen analysis and correlated TOP ODs to culture and AFB smear results (we did not perform a similar analysis in Boston because of the limited number of culture-positive specimens). For both studies, we

estimated per-patient sensitivity and specificity compared to culture as the reference standard using all available results to adjudicate TB status. We also estimated per-patient sensitivity and specificity using a composite reference standard (CRS) that included AFB smear, response to antituberculous treatment, mycobacterial culture and *M. tuberculosis* sequencing (e.g. 2-ponA genotyping), as recommended [17, 18, 30]. In Boston, we analyzed patient characteristics according to TOP and culture results using Wilcoxon test (for continuous data) and Fisher's exact test (for categorical data).

Ethical approvals. The studies were approved by the Comité de Ética em Pesquisa do Hospital Universitário Cassiano Antônio de Moraes and the Institutional Review Board at Boston University Medical Center. Samples were shipped to Boston for genotyping under a Material Transfer Agreement.

Results

Analytical specificity studies. TOP TB's primer set 3-ponA (used for diagnosis) demonstrated no cross-reactivity against a panel of 20 species of NTM (Figure 2). As shown, the TOP PCR product was amplified only with *M. tuberculosis* DNA and no targeted DNA sequences (185 bp) were amplified when using DNA from NTM species. The 2-ponA primer set (used for sequencing) demonstrated similar analytical specificity (data not shown).

Clinical studies. We evaluated the diagnostic accuracy of the TOP assay in two separate cohorts of subjects with suspected pulmonary TB in Vitória and Boston. In all, the study included a total of 132 specimens from 106 subjects (Figure 1). Supplementary Table 1 summarizes key design features of the two studies.

Brazil pilot study. From January 28 to April 11, 2014, we obtained 73 samples from 46 patients; 26 (36%) patients provided a single specimen, and 47 (64%) provided >1 sample. One sample was excluded (missing TOP result). The median age of patients was 35 years (range 10-76), 65% were male and the median sample volume was 1 mL (range 0·5-2). In a *per-specimen* analysis (Figure 2a), 37 (51%) samples were culture-positive, of which 34 were also TOP-positive. Twenty-three (32%) were negative for *M. tuberculosis* by both methods, including three (all smear AFB+) that grew NTM species (*M. abscessus, M. avium and M. kansasii*). Twelve (17%) samples (five smear AFB-positive and seven smear AFB-negative)

were TOP-positive but culture-negative (n=11) or contaminated (n=1). TOP results by culture and by AFB smear results are shown in Figures 2b and 2c, respectively.

In a *per-patient* analysis (n=46) that included all available results per participant (i.e. including both study and routine clinical care results), 22 (48%) patients were culture-positive, all of which were also TOP-positive, and 14 (30%) were negative on both tests. Of the remaining 10 (22%) patients with culture-negative/TOP-positive results, two had a culture-positive sister sample, and one had a clinical diagnosis of TB that improved after treatment (Supplementary Table 3). Of the other seven patients, 2pon-A genotyping confirmed the presence of *M. tuberculosis* DNA in six (one sample was unavailable). The clinical history of these seven individuals with discordant results is shown in Supplementary Table 4. The per-patient sensitivity and specificity of TOP in Brazil compared to culture vs. the CRS are shown in Table 1.

Boston study. Between February 21 and August 1, 2014, we obtained 60 respiratory samples (64% sputum, 35% induced sputum and 1% endotracheal aspirates) sourced from 60 patients. Supplementary Table 2 shows demographic characteristics of the study population. The median age of patients was 54 years (range 17-92), 63% were male, 50% were non-US born, 12% were HIV-infected, and 38% were current or former smokers. The median specimen volume was 1 mL (range 0·5-3). Of the 60 patients, 2 (3%) were culture positive; of these, one was TOP-positive (smear AFB-negative) and one was a bloody sample that was TOP-negative (smear AFB-positive). Fifty-two (87%) subjects tested negative on both tests, including three who were culture-positive for NTM (two *M. avium* and one *M. kansasii*). The remaining six patients were culture-negative/ TOP-positive, of whom two had a culture-positive sister sample (Supplementary Table 3); genotyping results confirmed *M. tuberculosis* DNA in all six (see Supplementary Table 4 for clinical histories). The sensitivity and specificity of TOP compared to culture in the Boston study vs. the CRS are shown in Table 1.

Discussion

This study adds to published evidence that the TOP TB assay accurately detects *M. tuberculosis* DNA in respiratory samples from individuals with suspected TB with otherwise negative TB results based on culture alone. In the absence of culture to determine specificity, we validated our results using a CRS that included sequencing *M. tuberculosis* directly from

specimens. Further, the analytical and clinical specificity of the assay demonstrate no cross-reactivity against closely related NTM species, further strengthening our results. These findings are consistent with both old and new data demonstrating the limitations of mycobacterial cultures to diagnose individuals with paucibacillary TB disease.

For decades, rapid detection of pulmonary TB has relied on sputum AFB smear microscopy, but its yield is low when compared to mycobacterial cultures [7]. Recently developed molecular tests such as Xpert MTB/RIF and GenoType MTBDRplus provide a rapid alternative to culture for patients with high bacterial loads (i.e. sputum smear AFB-positive). However, their overall sensitivity (~90% against culture) in programmatic conditions has been lower than initially anticipated [31], and particularly poor (~50%) in patients with paucibacillary TB disease [17, 32-34]. A newer and improved version of the Xpert MTB/RIF assay (e.g. Xpert MTB/RIF Ultra) has demonstrated improved sensitivity, but it still remains below that of culture [35]. Therefore, a critical diagnostic gap in many programmatic settings remains. Without microbiological confirmation, the diagnosis of paucibacillary TB disease is rarely definite, forcing clinicians to employ empirical treatment algorithms that lead to over- or under diagnosis, unnecessary drug toxicity, and increased morbidity and mortality [1, 36].

Results from this study add credence to our previous finding of 72% TOP-positivity (compared to 18% culture-positivity) in the sputum of HIV-infected patients with suspected pulmonary TB in Mbarara, Uganda [22] –a population at a particularly high pre-test probability of TB disease. The pilot study in Brazil demonstrated feasibility in predominantly sputum smear AFB-positive specimens but provided limited information on overall diagnostic performance. In contrast, diagnostic accuracy of TOP in Boston was almost identical to that reported in Uganda, despite extreme differences in disease prevalence. The clinical histories of patients with discordant culture-negative/ TOP-positive results in this study were highly suspicious for TB disease, as indicated by repeated evaluations for TB over time, individual risk factors for TB disease, and imaging findings consistent with TB. Furthermore, of the 16 individuals with discordant results, four had a previous or concurrent culture-positive sample or a clinical diagnosis of TB. Of the eight patients with culture-positive NTM disease, six were TOP-negative and two were TOP-positive. Based on the clinical history of the latter two patients, we suspect a mixed mycobacterial infection, where *M. tuberculosis* likely represented a minority population, as suggested in a recent study with Xpert MTB/RIF that

coincidentally included the same two countries as this study [37]. Finally, we used a reproducible genotyping method that is based on the genetic signature of a global collection of *M. tuberculosis* isolates representing all major phylogenetic lineages [22]. The variable frequency of 2-ponA variants between Mbarara, Vitória and Boston add yet another layer of evidence. Taken together, these data suggest that TOP TB enables accurate detection of *M. tuberculosis* DNA in several categories of culture-negative paucibacillary TB patients including those with HIV/AIDS, extra-pulmonary TB, and old untreated TB (as indicated by apical fibrous scarring on chest radiograph) [38, 39].

While promising, our results will require clinical validation with patient outcomes as the biological and clinical significance of detecting *M. tuberculosis* DNA in culture-negative TB suspects is not well understood and rife with speculation [40]. Currently, the most widely held opinion is that such results represent non-viable bacilli that are clinically insignificant because DNA-positive/culture-negative discordant results have been most frequently reported in individuals during or after completing antituberculous therapy [40-42]. However, several lines of evidence suggest that this interpretation may be an oversimplification, particularly when semi-quantitative assays such as TOP indicate trace amounts of *M. tuberculosis* DNA are present (i.e. confirming paucibacillary disease), or no previous history of TB treatment is elicited. First, mycobacterial culturing techniques were specifically developed several decades ago to provide a nutrient rich, oxygen and temperature-controlled medium artificially optimized for mycobacterial growth; therefore, their yield is susceptible to disruption by various conditions such as chemicals, antituberculous treatment, and as-yet poorly understood phenotypic or genotypic changes altering mycobacterial growth dynamics. For example, the sample decontamination process that is required prior to standard cultures is inherently detrimental to mycobacterial viability, likely compromising their yield in paucibacillary samples [7]. Second, *M. tuberculosis* is known to enter into a persister-like phenotype shortly after initiation of antituberculous treatment. Although *M. tuberculosis* with the persister phenotype is non-culturable with standard culture media, its viability is restored with enriched media [9, 43]. Lastly, there is extensive precedent, extending back over 20 years, of numerous molecular assays with analytical and clinical sensitivity that is superior to that of culture for multiple microorganisms and sample types [44]; early results for many of these tests faced similar claims of non-viability, only to later replace culture as the preferred diagnostic method [45, 46].

Furthermore, these is a growing body of data challenging the notion that mycobacterial growth is required to define clinically relevant TB disease. In a study that used advanced imaging techniques, patients with initially culture-positive results who remained persistently Xpert-positive at end of antituberculous treatment (even when clinical and microbiological cure was achieved), were at increased risk for TB relapse [8]. In South Africa, Chengalroyen et al recently confirmed the existence of differentially detectable *M. tuberculosis* populations in the sputum of patients with suspected TB that were not culturable with standard mycobacterial culture methods [9, 10]. Other individuals with active disease harboring non-culturable organisms include those with unstable latent TB infection or persons with early sub-clinical disease who have "percolating" organisms [47], and those with old untreated TB [39]. Taken together, these data have significantly complicated the TB diagnostic landscape and show the need for caution when interpreting DNA-positive/ culture-negative results in patients with a high-pretest probability of disease [11].

Limitations

Our study has limitations. The two studies reported here used minor differences in sample handling and laboratory methods, which may have introduced some measure of variability in assay performance when compared to culture. In Boston, there were a small number of culture positive samples; and overall, four of the specimens we tested were culture-positive/TOP-negative (three of which had a TOP-positive sister sample) as a result of including challenging specimens such as low-volume samples and one hemorrhagic sample in Boston (PCR inhibition could have led to a false-negative result). Also, the colorimetric readouts used different, study-specific cut-off values to establish the "Limit of Blank", a key assay parameter [29]. Future development work will need to incorporate the TOP assay into a standardized molecular platform. Importantly, we did not follow participants with TOP-positive/culture-negative results for clinical outcomes; therefore, we do not know the clinical significance of such results. Admittedly, detection of trace amounts of *M. tuberculosis* DNA may be due to bacterial "spillage" from a dormant lung focus or low-level bacterial replication that may not require treatment. Finally, the TOP TB assay does not include provisions for detecting drug-resistant TB, but the primary global need is for a rapid and reliable triage test with high sensitivity [2, 6].

Conclusions

Because of limitations in existing technologies, including mycobacterial cultures, microbiological confirmation of paucibacillary TB disease remains a key diagnostic gap. By shifting diagnostic emphasis to early detection, the TOP TB assay broadens sensitive and accurate detection of *M. tuberculosis* across the clinical spectrum of pulmonary TB disease. While use of the TOP assay for definitive diagnosis of active disease will require prospective validation with clinical outcomes, its operating characteristics suggest that it may have more immediate utility as a triage or "TB rule out" test.

Table 1: Per-patient sensitivity and specificity of the TOP TB assay according to a reference standard established by *Mycobacterium tuberculosis* culture or a Composite Reference Standard in Brazil and Boston studies.

Study site	Culture Reference Standard				Composite Reference Standard [a]			
	MTB detected	MTB not detected	Sensitivity (95% CI)	Specificity (95% CI)	MTB detected	MTB not detected	Sensitivity (95% CI)	Specificity (95% CI)
Brazil (n=46)								
TOP TB assay	22/22	14/24	100% (85-100)	58% (37-77)	31/31	14/15	100% (89-100)	93% (68-100)
Culture	–	–	–	–	22/31	15/15	65% (46-80)	100% (78-100)
Boston (n=60)								
TOP TB assay	1/2	52/58	50% (1-99)	90% (78-96)	7/8	52/52	88% (47-100)	100% (93-100)
Culture	–	–	–	–	2/8	52/52	25% (3-65)	100% (93-100)

Definition of abbreviations: CI= Confidence interval; MTB= *Mycobacterium tuberculosis*

[a] Composite Reference Standard (CRS) included *M. tuberculosis* culture, response to antituberculous treatment, AFB smear and *M. tuberculosis* sequencing (e.g. 2-ponA genotyping).[17] The breakdown of CRS results is shown in Appendix (Table S2)

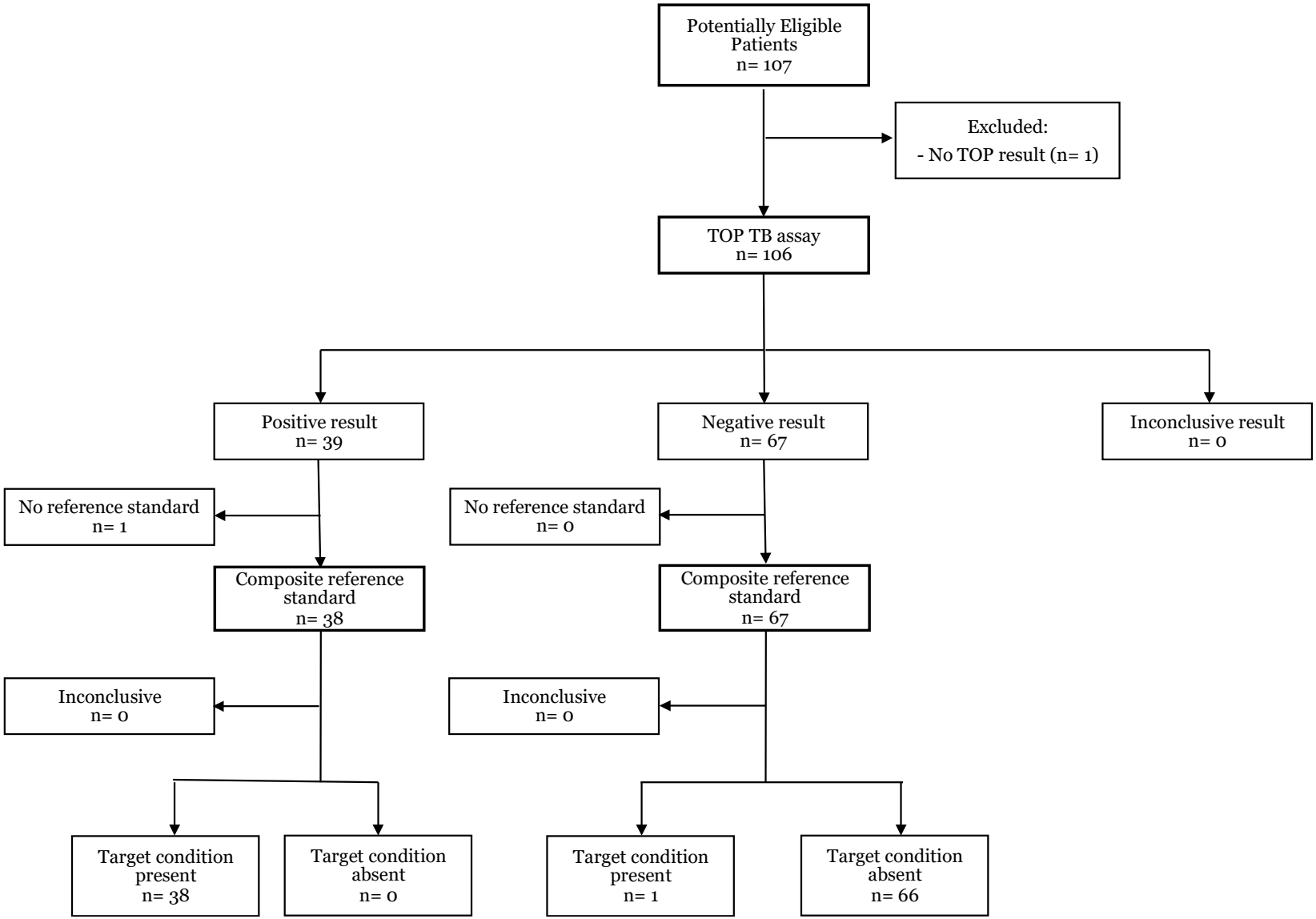
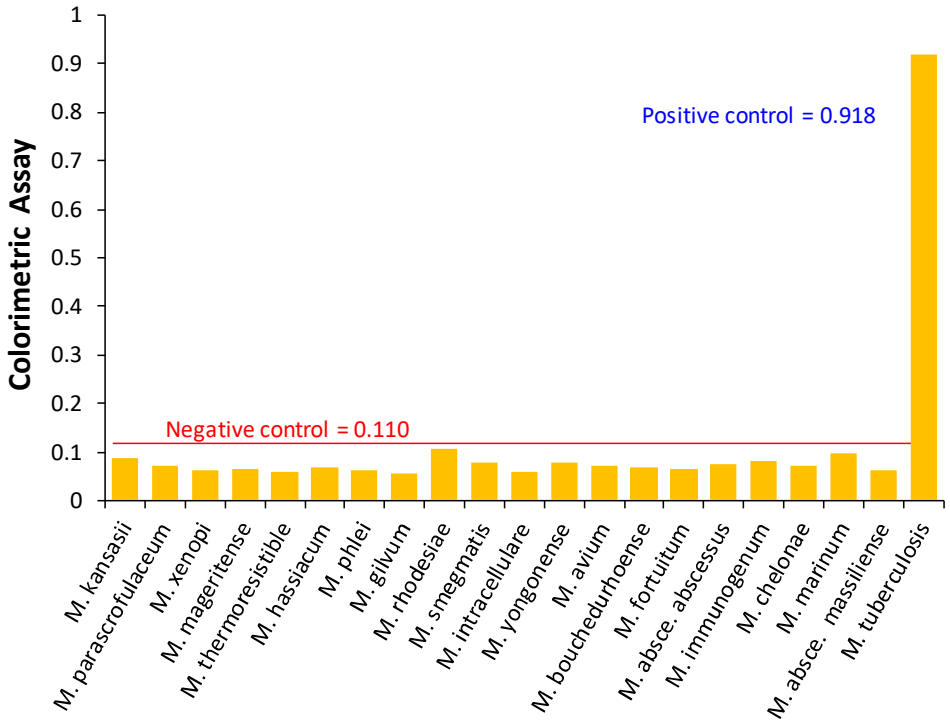

Potentially Eligible Patients
n= 107
Excluded:
- No TOP result (n= 1)
TOP TB assay
n= 106
Positive result
n= 39
Negative result
n= 67
Inconclusive result
n= 0
No reference standard
n= 1
No reference standard
n= 0
Composite reference standard
n= 38
Composite reference standard
n= 67
Inconclusive
n= 0
Inconclusive
n= 0
Target condition present
n= 38
Target condition absent
n= 0
Target condition present
n= 1
Target condition absent
n= 66

Figure 1



TOP OD	Mycobacterial Species	Culture ID
0.089	M. kansasii	MK-1037
0.070	M. parascrofulaceum	MO-5495
0.061	M. xenopi	MO-5452
0.065	M. mageritense	MO-5569
0.060	M. thermoresistible	MO-3080
0.067	M. hassiacum	MO-5426
0.063	M. phlei	MO-16:53109
0.057	M. gilvum	MO-3761
0.108	M. rhodesiae	MO-5403
0.078	M. smegmatis	MO-5511
0.060	M. intracellulare	MA-16:53588
0.078	M. yongonense	MA-7437
0.071	M. avium	MA-8495
0.068	M. bouchedurhonense	MA-6521
0.066	M. fortuitum	MF-4621
0.075	M. abscessus sub. abscessus	MC-10021
0.080	M. immunogenum	MO-5515
0.071	M. chelonae	MC-16:53526
0.098	M. marinum	MM-780
0.061	M. abscessus sub. Massiliense	MC-16:53493
0.918	M. tuberculosis	BCG
0.110	Negative control	N/A

Figure 2

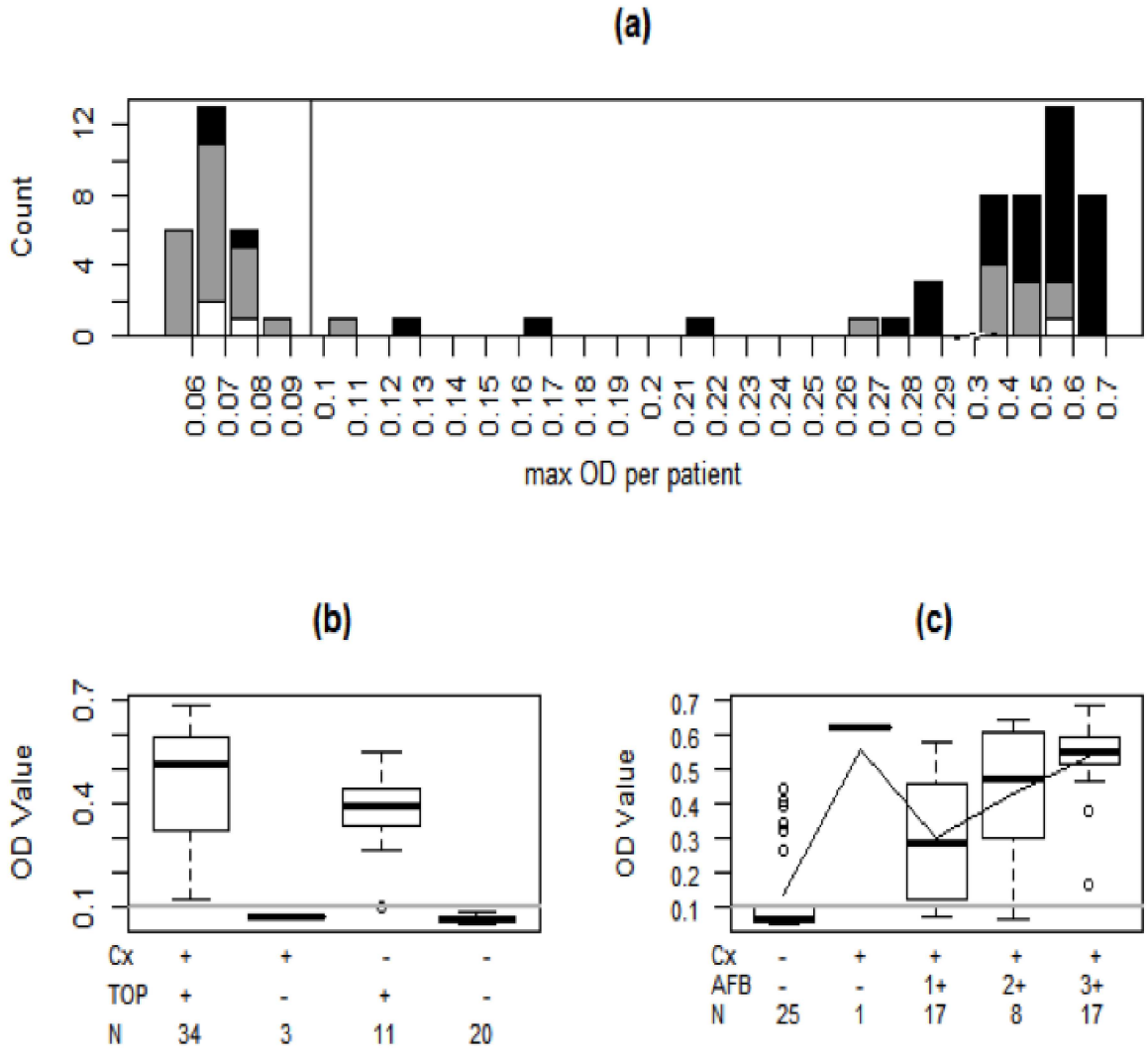

(a)
Count
0
4
8
12
0.06
0.07
0.08
0.09
0.1
0.11
0.12
0.13
0.14
0.15
0.16
0.17
0.18
0.19
0.2
0.21
0.22
0.23
0.24
0.25
0.26
0.27
0.28
0.29
0.3
0.4
0.5
0.6
0.7
max OD per patient
(b)
OD Value
0.1
0.4
0.7
Cx + + - -
TOP + - + -
N 34 3 11 20
(c)
OD Value
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Cx - + + + +
AFB - - 1+ 2+ 3+
N 25 1 17 8 17

Figure 3

Figure captions:

Figure 1: Study profile.

Figure 2: Analytical Specificity of the TOB TB Assay against Selected Non-Tuberculosis Mycobacteria (NTM). This panel includes 20 different species of NTM provided by Richard Wallace, MD (University of Texas at Tyler, TX). The list of species was selected by their genetic proximity to TOP TB's diagnostic target (3ponA). For each NTM species, a loopful of culture was harvested into DNA extraction solution (Epicentre, Madison WI) and processed for DNA extraction as described [22]. DNA preparations (~10^5 to 10^7 colony-forming units; high copy numbers) were mixed with human DNA preparation (0.5 micrograms from a healthy volunteer) before testing to simulate clinical sample conditions. Positive (~25 CFU of *M. tuberculosis*; low copy number) and negative controls containing 0.5 micrograms of background human DNA were included. TOP TB assay conditions and primers were used to generate amplicons that were detected using the same capture-probe colorimetric assay used for the TOP TB assay, with resultant Optical Densities (OD) [22].

Figure 3: TOP TB assay results in 72 specimens (46 subjects) from Vitória, Brazil. Four subjects with nontuberculous mycobacteria were excluded from graphs b and c for plotting purposes. **(a)** Vertical line denotes the cut-off TOP OD (0.0984) for a positive test. Histograms represent the number of subjects with culture-positive (black), culture-contaminated (white) and culture-negative (grey) results, by TOP OD values. The X-axis is zoomed in at the lower end of TOP OD values (0.100 to 0.300) for ease of comparison with the Uganda study [22]. **(b)** Group TOP OD values according to culture (Cx) and TOP results (group means are 0.47, 0.07, 0.38 and 0.06, from left to right). The mean TOP OD of culture-positive/TOP-positive (0.47) and culture-negative/TOP-positive samples was similar (0.38, P=0.07). **(c)** Median TOP ODs paralleled sputum acid-fast bacilli (AFB) smear microscopy grades (Kruskall Wallis P<0.001), supporting semi-quantitative performance of the TOP TB assay. A smoothing spline fit to the data is shown. When compared to culture, the per-specimen sensitivity and specificity of the

TOP TB assay in Brazil was 92% (34/37, 95% CI 77%–98%] and 66% (23/35, 95% CI 48-80), respectively.

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Supplementary Data.

Standard laboratory methods.

Brazil. Sputum samples were delivered to the NDI laboratory per routine clinical care. Upon arrival, specimens were prepared and examined for AFB smear microscopy (auramine O fluorescent stain) according to established guidelines [48]. For culturing mycobacteria, we followed the method described by Kudoh [49]. Briefly, the sputum was placed on a swab and immersed in a tube of 4% NaOH solution for 2 min, and then spread on the surface of Ogawa-Kudoh medium. The inoculated media containers were incubated at 37°C, and mycobacterial growth was evaluated weekly for up to 8 weeks. Positive specimens were stained to confirm the presence of AFB organisms, and *M. tuberculosis* identified using phenotypic tests [50].

In Brazil, once the routine work was completed, if the remaining volume of the sample was ≥1 mL, it was coded and stored unprocessed in a refrigerated cabinet until for up to 1 week (as per routine clinical care). After TOP processing, extracted DNA was then frozen at -80°C for two months until TOP testing in a single batch at the NDI without knowledge of culture results.

Boston. Respiratory specimens were delivered to the BMC Microbiology Laboratory per routine clinical care. Upon receipt of samples, a 2 mL aliquot was processed for routine mycobacterial stains and cultures using Alpha Tech's NAC-PAC EA3 digestant/ decontamination kit, and then inoculated on a Lowenstein Jensen (LJ) slant, a BBL Mitchison 7H11 slant and into a Versatrek ESP Myco bottle along with 2 smears for AFB staining using the Scientific Devices' Rapid Modified Auramine O stain. The liquid medium was then inserted into the Versatrek automated cabinet for incubation (total 6 weeks unless signal positive growth was confirmed earlier). The LJ and the 7H11 slants were inserted into an incubator held at 35-37C in 5-10% CO2 for a total of 8 weeks or until culture growth was confirmed earlier. Positive cultures were then stained to confirm the presence of AFB organisms. After smear confirmation, the isolate or the broth was tested using both GenProbe/ AccuProbe *M. tuberculosis* complex and *M. avium* complex Probe kits. If the results were negative, an aliquot was inoculated into

Middlebrook 7H9 broth and incubated until growth was consistent with a 1.0 McFarland turbidity standard, then the broth was retested using both AccuProbe kits. If the results were repeatedly negative, the isolates were sent to a reference laboratory (ARUP, Salt Lake City, UT) for further identification.

In Boston, once the routine work was completed, if the remaining volume of the sample was ≥1 mL, it was coded and stored unprocessed in a refrigerated cabinet for up to 1 week (as per routine clinical care). Eligible samples were picked up weekly and subjected to the TOP sample processing method within 24-48 hours after receipt in the research laboratory. TOP testing was done in weekly batches without knowledge of smear and cultures results.

667 **Supplementary Table 1**: Summary of clinical studies.

	Brazil (pilot study) N=46	Boston N=60
Study design	Cross-sectional, convenience sample of consecutive sputum specimens (N=72)	Cross-sectional, convenience sample of consecutive specimens with a laboratory request for *M. tuberculosis* testing
Patients	Ambulatory, mostly adult individuals with suspected TB. Of the 6 patients with known HIV results, 1 (17%) was infected	Hospitalized and ambulatory adult patients with suspected TB (12% HIV-infected)
Specimen type and handling	Discarded fresh respiratory specimens[1]. Specimens were tested in a single batch in Brazil	Discarded fresh respiratory specimens [2]. Specimens were tested in weekly batches in Boston
Reference method(s)	Solid culture (Ogawa-Kudoh)	Lowenstein Jensen and Middlebrook 7H11 solid cultures, and Versatrek ESP Myco liquid culture
Other clinical and microbiological data available	Review of medical records and results from sister sputum samples tested by the local TB program	Review of computerized medical records

668 [1] Respiratory specimens included spontaneously expectorated sputum, induced sputum, and
669 bronchoalveolar lavage or washing
670
671

672 **Supplementary Table 2**: Characteristics of 60 Individuals with Suspicion of Pulmonary Tuberculosis Disease in
673 Boston, Massachusetts According to *Mycobacterium tuberculosis* Culture and TOP TB Assay Results.

Characteristic	Overall n=60	*M. tuberculosis* detected [a] n=8	*M. tuberculosis* not detected n=52	P value [b]
Age				
Median [IQR]	54 [40, 63]	61.5 [54.4, 69.5]	52.5 [38, 61]	0.13
Mean (SD)	52.4 (17.0)	59.5 (15.3)	51.3 (17.2)	
Range	17-92	28-77	17-92	
Male	38 (63)	5 (63)	33 (63)	1.0
Non-US born	n=51	n=7	n=44	0.69
	30 (50)	5 (63)	25 (48)	
Race/ Ethnicity	n=59	n=8	n=51	0.87
White/ Caucasian	12 (20)	1 (12)	11 (21)	
Black/ African American	22 (37)	3 (38)	19 (37)	
Hispanic/ Latino	9 (15)	2 (25)	7 (13)	
Other	16 (27)	2 (25)	14 (27)	
Smoking history	n=37	n=6	n=31	0.47
Current	11 (18)	2 (25)	9 (17)	
Former	12 (20)	3 (37)	9 (17)	
Never	14 (23)	1 (12)	13 (25)	
Alcohol use	7 (12)	1 (13)	6 (12)	1.0
Homeless	7 (12)	1 (13)	6 (12)	1.0
Veteran	1 (2)	0 (0)	1 (2)	1.0

Medical insurance				0.26
Medicare	6 (10)	0 (0)	6 (12)	
Medicaid	34 (57)	7 (88)	27 (52)	
Other	20 (33)	1 (13)	19 (37)	
HIV infected	7 (12)	1 (12)	6 (12)	1.0

674 Values are median [IQR, interquartile range] or number (percentage), unless otherwise specified

675 Definition of abbreviations: AFB= Acid-fast bacilli; HIV= Human immunodeficiency virus; US= United States

676 [a] Culture+/ TOP+ (n=1); culture+/ TOP– (n=1); Culture–/ TOP+ (n=6)

677 [b] P-values calculated using either Fisher exact test or Wilcoxon test

678

679

Supplementary Table 3: Breakdown of results for the Composite Reference Standard (CRS) in 106 pulmonary tuberculosis suspects in Vitória (Brazil) and Boston (U.S.A.).

Composite Reference Standard Criterion				TOP TB assay	CRS interpretation	N
1	2	3	4			
Culture	Sequencing[1]	Response to TB treatment	AFB smear			
Brazil						46
+	Not done	Not available	+/–	+	MTB detected	22
+*	Not done	Not available	–	+	MTB detected	2
–	Not done	+	–	+	MTB detected	1
–	+	No treatment	+/–	+	MTB detected	6
–	Not Available	No treatment	–	+	Incomplete CRS	1
–	Not done	NTM treatment	+	–	MTB not detected	3
–	–	No treatment	–	–	MTB not detected	11
Boston						60
+	Not done	Not available	+	–	MTB detected	1
+	Not done	Not available	–	+	MTB detected	1
+*	+	Not available	–	+	MTB detected	2
–	+	Not available	–	+	MTB detected	4
–	Not done	NTM treatment	+	–	MTB not detected	3
–	Not done	No treatment	–	–	MTB not detected	49
					Total	106

682 Definition of abbreviations: AFB= Acid-fast bacilli; MTB= *M. tuberculosis*; NTM= Nontuberculous mycobacteria; TB= Tuberculosis

683 *Sister sample from outside of study was culture-positive for *M. tuberculosis* (Brazil N=2, Boston N=2)

684

Supplementary Table 4: Clinical summaries of individuals suspected of tuberculosis disease with discordant culture−/TOP+ results in Brazil and Boston. The presence of *M. tuberculosis* DNA in culture-negative samples was confirmed by 2-ponA genotyping.

ID	Clinical description	TOP OD	2ponA genotype
	Brazil [1]		
1/60	35/F with systemic sclerosis, pulmonary hypertension and previous history of pulmonary TB presented for evaluation because of persistent cough in January 2014. She had been treated for sputum (AFB 3+) culture-positive pulmonary TB from August 2008 to March 2009. In December 2008, she was diagnosed with systemic sclerosis and was treated with cyclophosphamide pulses. Her respiratory function deteriorated slowly and was diagnosed with pulmonary hypertension secondary to systemic sclerosis. In January 2014, she was evaluated for TB (two sputum samples, both AFB and culture-negative; 1/2 was TOP positive OD=0.268, genotype 3). At that time, she was treated for acute trachea-bronchitis (7 days, levofloxacin). Patient was again treated for tracheo-bronchitis in May 2014 (10 days, cefepime) and July 2014 (5 days, azithromycin). In August 2014 she was seen as an outpatient in the pulmonary clinic where she was again evaluated for TB (AFB and culture-negative).	0.268	3
2/62	45/F with HIV infection diagnosed in 2010 with irregular antiretroviral treatment (CD4 cell count and HIV viral load not available), rheumatoid arthritis, fibromyalgia and regular crack cocaine use presented for evaluation in January 2014 for persistent cough; patient attributed her cough to her cocaine use. She had been evaluated for TB several times over the last four years because of a history of intermittent cough and fever. Between November 2010 and January 2014, she had 7 sputum samples for TB (all AFB and culture-negative). Her TST was 15 mm (2010). One sputum sample from January 2014 (AFB and culture-negative) was TOP positive (OD=0.392, genotype 2).	0.392	2

	In March 2014, she had a chest computed tomography with contrast that showed bilateral apical pleural scarring, pleuro-parenchymal fibrous bands of right middle lobe and both lower lobes, and a small area of ground-glass opacities in left lower lobe.		
3/66	17/F patient was evaluated for TB in January 2014. She had been admitted to a local hospital in December 2013 for unclear illness. Since her hospital discharge, she had remained unwell with intermittent fever and weight loss. No known TB contacts. In January 2014 she had three sputum samples (all AFB and culture-negative; 1/1 was TOP-positive, OD=0.324, genotype 3).	0.324	3
4/67	58/M evaluated for TB in January 2014. Clinical information for this patient was not available. In reviewing laboratory files, this patient had been evaluated for TB in September 2003 (two samples) and in April 2004 (two samples). All of his results to date were AFB and culture-negative. One sample tested in January 2014 was borderline TOP-positive (OD=0.103); this sample was not available for 2-ponA genotyping.	0.103	NA
5/73	38/M with no significant past medical history was evaluated for TB in February 2014 because of a 15-day history of productive cough, fever and diaphoresis. He had no known TB contacts. His TST was 5mm and a chest X-ray showed "scaring" (no further information was available). Three sputum samples were obtained (all AFB and culture negative; 1/2 samples tested was TOP-positive, OD=0.408, genotype 3). Patient symptomatically improved without antituberculous treatment.	0.408	3
6/84	63/M with epilepsy, ETOH abuse, glaucoma, peptic ulcer and history of piloroplasty and gastro-duodenal anastomosis (June 2011) was evaluated for TB in February 2014. Two sputum samples were obtained (both were AFB and culture negative; 1/1 sample tested was TOP-positive, OD=0.445, genotype 3)	0.445	3
7/106	72/F woman with glaucoma and macular degeneration referred to outpatient TB clinic for evaluation of pulmonary NTM infection in March 2014 because of a 3-year history of productive	0.518	2

	cough, low-grade fever, diaphoresis and 10 Kg weight loss. Her TB history dated back to June 2011 when she was first evaluated for TB; at that time, she was found to be sputum AFB-positive and started on antituberculous therapy. However, her culture grew *M. abscessus* and her treatment was modified. At that time, her chest X-ray showed reticulo-nodular infiltrates in both upper lobes. She had no known TB contacts. Because of persistent symptoms, between Jun 2011 and March 2014, she had been investigated for TB with 23 sputum samples; 7 (all AFB-positive) of these samples grew *M. abscessus*. In May 2012, she had a pleural biopsy and pleural fluid was sent for TB culture (both negative). A sputum sample (AFB 2+) from March 2014 that was culture-positive for *M. abscessus* was also TOP-positive (OD= 0.518, genotype 2).		
	Boston [2]		
1/1	52/F from Peru with no past medical history was diagnosed with smear-negative, culture-positive pulmonary TB in December 2014 and started on standard antituberculous treatment. At her 2-month follow-up visit, sputum samples were ordered; AFB and cultures were negative (one of these samples was borderline TOP-positive (OD=0.100, genotype 0^T). A chest X-ray showed a right upper lobe opacity that was improving, compared to prior exams.	0.100	0^T
3/15	57/M from Antigua was admitted to the hospital in April 2014 for epistaxis. His past medical history included multiple myeloma, a coagulation disorder and chronic renal failure. He was a current smoker. On admission, a chest radiograph showed right middle lobe opacity, bilateral pleural effusions and lytic lesions in clavicles and ribs. A chest CT showed multifocal airspace opacities and right upper lobe tree-in-bud infiltrate (radiological interpretation= “suspicious for TB”); three sputum samples obtained in separate days were AFB and culture negative (1/1 samples was TOP-positive, OD=0.203, genotype 2). Bacterial urine and blood cultures were negative, and a bone marrow aspirate was negative for AFB and cultures. TST and IGRA results during admission were negative. Patient was treated for possible aspiration pneumonia.	0.203	2

4/22	28/F from Vietnam with no past medical history was referred in April 2014 to the outpatient TB clinic for evaluation and treatment. A chest X-ray taken at that time showed a moderate right pleural effusion, small left pleural effusion, ill defined reticulo-nodular markings in the left apex and patchy opacities in the right apex. A sputum sample was obtained for AFB smear and culture (AFB and culture-negative; TOP-positive; OD=0.805, genotype 0^T). One month prior to referral, patient had undergone a diagnostic thoracocentesis at another hospital and a culture of her pleural fluid was positive for *M. tuberculosis* after 4 weeks of incubation.	0.805	0^T
5/27	64/M from Bangladesh was admitted to the hospital in May 2014 because of weakness related to having missed a hemodialysis session. He was a former smoker. His past medical history included diabetes mellitus, hypertension, gout, hyperlipidemia, cardiac arrhythmias, and end-stage renal disease on hemodialysis. His TB history dated back to February 2013 when he was referred to the TB clinic as part of a pre-renal transplant evaluation when he was found to have a positive TST (15 mm) and positive IGRA. A chest CT at that time showed mediastinal lymph node enlargement for which he had a broncholalveolar lavage (BAL) and transbronchial biopsy in March 2013. All cultures (BAL and lymph node) were negative for *M. tuberculosis* and the lymph node pathology was negative for TB adenitis. He was reevaluated for active TB during his May 2014 admission, when he had one induced sputum sample that was negative for AFB and culture (TOP-positive, OD=0.918, genotype 1). In June 2014, he was offered LTBI treatment but the patient declined.	0.918	1
6/41	63/M African-American from Boston was admitted in June 2014 for evaluation of weight loss and an abnormal chest CT. His past medical history included HIV (CD4= 731 cells/mL, viral load <75 copies/mL, compliant on antiretroviral therapy), HCV-associated liver cirrhosis and depression. He was a current smoker. A chest CT performed a few days prior to admission showed a thick, speculated, walled right upper lobe 4.8 cms cavitary lesion, multiple local nodular cavitary foci in right lung as well as left pulmonary nodules. His TB history included a chest X-ray (August 2005)	0.550	2

	that showed bilateral apical scarring and minimally elevated bilateral hila likely secondary to retraction (radiological interpretation = "findings are consistent with old TB"). In March 2009, he had a TST placed but he did not return for reading. In June 2014, a bronchoalveolar lavage (BAL) and two sputum samples were AFB 2+ and positive for *M. malmoense* (6-9 days to grow) (1/1 sample was TOP-positive, OD=0.550, genotype 2); another of the BAL samples was AFB-negative and had late growth (3 weeks) of *M. abscessus*. A sputum sample was Xpert Mtb/RIF negative. Bacterial urine and blood cultures were negative. The patient was treated with rifampin, ethambutol and azithromycin.		
7/46	60/M Caucasian man from Boston was admitted in June 2014 for COPD exacerbation and possible aspiration pneumonia. His past medical history included diabetes mellitus, hypertension, COPD, bronchiectasis, hepatitis B and C infection, and MRSA infection. He was a former smoker with a history of homelessness, alcohol and opioid abuse (on methadone). On admission, his imaging studies showed a right middle-lobe opacity (chest X-ray) and mediastinal and hilar lymph nodes up to 11mm (stable since 2013) and scattered tree-in-bud opacities (chest computed tomography). His TB history included a positive TST (size unknown) in 1994 for which he received treatment with isoniazid (6 months); the patient had been placed in respiratory isolation three times in the past 14 months for suspicion of pulmonary TB (April 2013, May 2013 and April 2014). During his admission, two spontaneously expectorated and two induced sputa were AFB and culture-negative (1/1 was TOP-positive, OD=0.340, genotype 2). Bacterial urine and blood cultures were negative and a separate sputum sample grew MRSA. The patient was treated with antibiotics. Unbeknown to us, this patient had provided a prior sputum sample in April 2014 that was also tested for TOP as part of a separate study (AFB and culture negative; TOP-positive, OD= 0.947, genotype 2).	0.340	2
8/49	77 /M from Dominican Republic was admitted to the hospital for evaluation of hemoptysis. His		

	past medical history included Wegener's granulomatosis, diabetes mellitus, hyperlipidemia and end-stage renal disease on hemodialysis. On admission his chest X-ray showed left patchy opacities. The patient's chart did not have TST or IGRA results and no previous TB history. Two sputum samples were AFB-positive (2+ and 4+) and grew *M. tuberculosis* (1/1 sample was TOP-negative, OD=0.075; 2ponA genotyping was not performed) and the patient was started on antituberculous treatment. The discarded portion of the sample available for TOP testing was bright red blood, a well-known PCR inhibitor.	0.075	ND

NA= Not available; ND= Not done

Clinical summary:

[1] Brazil: Of the 10 patients (12 samples) with culture-negative/ TOP-positive results, *M. tuberculosis* was confirmed in three patients (all sputum AFB+ smear) either microbiologically (two patients had a culture-positive sister sample) or clinically (chest X-ray and response to treatment). One sample (AFB+) that grew *M. abscessus* was from an elderly woman with a clinical history that was consistent with a mixed mycobacterial infection, and one patient had completed treatment for culture-positive pulmonary TB ~5 years before this study. The other five patients shared risk factors for TB disease (HIV infection, gastro-duodenal surgery) or symptoms and signs consistent with TB disease present at the time of testing. 2ponA genotyping results confirmed the presence of *M. tuberculosis* DNA in 6/7 available samples, including the patient with suspected mixed mycobacterial infection.

[2] Boston: Six patients (one sputum AFB+ smear and five sputum AFB– smear) were TOP positive but culture-negative; of these, TB was microbiologically confirmed in two patients (culture-positive sister samples). Another AFB+ sample that grew *M. malmoense* in culture was from an HIV-infected man with radiographic evidence of old TB disease (apical scaring). The other three patients shared traditional risk factors for TB in the U.S. (non-US born, homelessness and

703 immunosuppression), and two of them had been evaluated several times for active TB in the past 1-2 years. 2ponA
704 genotyping results confirmed the presence of *M. tuberculosis* DNA in all six samples.
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